Knock-down of the Golli Myelin Basic Protein in M3 Enhancer Knock-out Mice Causes the Dysregulation of Embryonic Stem Cell Differentiation

Research Thesis

Presented in partial fulfillment of the requirements for graduation with research distinction in Neuroscience in the college of Arts and Sciences of The Ohio State University

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April 2016

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Abstract

During development, in the central nervous system (CNS), oligodendrocyte progenitor cells (OPCs) migrate to axons and eventually become mature oligodendrocytes. Mature oligodendrocytes wrap around axons and produce myelin sheaths, which insulate the axons. Growth factors, specifically platelet derived growth factor (PDGF) and fibroblast growth factor (FGF), are released in the development of OPCs to promote congruent gene expression. PDGF is released to induce the proliferation of OPCs, and to promote their migration to axons. FGF induces OPC maturation into oligodendrocytes, and initiates axon myelination. OPCs act in a similar fashion following a spinal cord injury. PDGF and FGF are released as new OPCs develop, migrate to the injury site, and mature into oligodendrocytes in an attempt to repair damaged axons. We observed the same processes occurring *in vitro* with embryonic stem cells (ESCs) from our wild-type mouse model. Growth factors FGF and PDGF were added to the media to promote OPC morphology in mature ESCs by the activation of their respective growth factor receptors leading to the phospholipase C-gamma (PLCγ) pathway which results in the Ca^{2+} efflux from the endoplasmic reticulum (ER). In 1993, the Campagnoni lab discovered that the *myelin basic protein* (*mbp*) gene was part of a larger transcription complex called *golli-mbp*, whose products regulate store operated calcium channels on the ER membrane. We were able to better study the effects of golli-MBP through our M3 enhancer knockout (M3KO) mouse model where golli-MBP products were downregulated at the OPC stage of development. Deletion of the M3 enhancer resulted in the down regulation of both golli and MBP specific proteins *in vivo*. We generated WT and M3KO ES cell lines to investigate the potential influence of Golli-MBP in progenitor cell maturation. We found that the M3KO ES cells did not respond to FGF or PDGF to allow for proper cell adhesion and the differentiation into OPCs. These results suggest that deletion of the M3 enhancer reduces the expression of golli-MBP, which leads to the dysregulation of the signaling pathways necessary for the differentiation of ES cells into OPCs.

Introduction

Stem cells can be totipotent, pluripotent, multipotent, oligopotent, or unipotent depending on the tissue origin of the progenitor cell (Temple, 2012). Embryonic stem cells (ESCs) are pluripotent cells that are characterized by their capacity for self-renewal and their ability to generate all cell types of the ectoderm, mesoderm, and endoderm while in an undifferentiated state (Yu and Thomson, 2010). Because of these properties, ESCs are widely used in research due to their relatively unlimited supply, and their ability to differentiate into a plethora of cell types. The functions and processes of ESCs are regulated by multiple intracellular and extracellular agonists, including the release of growth factors, which induces a broad range of cell-type specific signaling events. *In vitro*, chemical inducers, such as these growth factors and chemokines, are added to the cell culture to promote proliferation, survival, and differentiation (Annerén, 2008).

Growth factors interact with target receptors to induce specific cellular responses to stimuli. Examples of growth factors pertinent in ES studies include Leukemia inhibitory factor (LIF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and platelet-derived growth factor (PDGF). LIF is a cytokine that modulates the proliferation of ES cells and stimulates self-renewal processes through activation of the Janus kinase/ signal transducer and activator of transcription 3 (JAK/STAT3) and the extracellular regulated kinase 1/2 (ERK1/2) signaling pathway (Annerén, 2008). Activation of JAK, by the LIF receptor, induces the phosphorylation of the STAT3 transcription factor. Upon phosphorylation, STAT3 dimerizes and migrates into the nucleus where it binds to promotor regions of genes regulating self-renewal. *In vitro*, researchers utilize the activity of LIF to inhibit the differentiation of the ESCs. bFGF acts on a variety of FGF receptors, including FGFR4, which induces differentiation by the termination of the ES self-renewal program primarily through the activation of the ERK1/2 signaling pathway (Ying et al., 2008). These studies suggest that the ERK1/2 signaling cascade plays a role in the ESC's commitment to a cell lineage. Upon binding bFGF, the receptor homo-dimerizes and cross-phosphorylates specific

tyrosine residues on the partnering monomer. This allows the Son of Sevenless (SOS) guanine nucleotide exchange factor to initiate the phosphorylation cascade by activating Ras and up-regulating receptor tyrosine kinase activity (Mebratu et al., 2009).

Protein kinases give regulate very specific functions in the cell through the phosphorylation and activation of target kinases and proteins. ERK1/2 is a mitogen-activated protein kinase (MAP kinase) that regulates the growth and proliferation of many cell types (Fyffe-Maricich et al., 2011), including ESCs. ERK1/2 acts via the MAP kinase I signaling cascade that plays a major role in cell metabolism, adhesion, migration, survival, and differentiation. Extracellular stimuli, particularly growth factors, may interact with receptor tyrosine kinases to initiate two different signaling pathways. Growth factors can not only trigger the MAP Kinase cascade, but activate the phospholipase C-gamma (PLC-γ) pathway as well (Moenning et al., 2009). The PLC-γ pathway acts through store operated calcium channels (SOCCs) causing a Ca^{2+} efflux from the endoplasmic reticulum (ER) (Markova et al., 2010).

The activation and roles of growth factors change depending on environment and cell type. The timing of growth factor receptor activation in ESCs are similar in the development and maturation of oligodendrocyte progenitor cells (OPCs) in the central nervous system (CNS). OPCs are glial cells in the central nervous system that are fated to become mature oligodendrocytes. Post-natally, in mice, PDGF release induces OPC proliferation and migration to axons (Zhu et al., 2014). FGF release induces their development into immature oligodendrocytes, and then further into myelinating oligodendrocytes (Fortin et al., 2005). FGF is also present when mature oligodendrocytes wrap around axons to promote the production of myelin sheaths (Furusho et al., 2012). This insulates the axons to promote maximal signal transduction for communication between neurons. The generation of myelin is most important early in life and steadily declines throughout maturity. Although myelin basic protein (MBP) is not as highly expressed throughout adult life, oligodendrocytes are stimulated to generate more myelin as a result of demyelination following traumatic injury (Zawadzka et al., 2010). Spinal cord injuries mirror

early developmental events, in that FGF and PDGF are released as OPCs are recruited to develop and migrate to the site of injury in an attempt to re-myelinate damaged axons.

Spinal cord injuries trigger a cascade of intracellular and extracellular events that lead to changes in cell function and expression (D'Angelo, 2013). Currently, spinal cord injuries present a wide range of neuropathologies as well as limited functional recovery. Much damage is due to secondary processes that result from the sudden influx of cytokines, such as TNF-α and IL-1β, chemokines, and the inflammation that follows through microglia activation (Rivest, 2011). Although cytokines are important for recovery, their sustained increase can lead to neuronal death. Such pathologies may include glial scars due to astrocytic and microglia activation, dystrophic neurons, neuronal cell death, secondary axon damage, axon demyelination, and delayed Ca^{2+} influx (Oyinbo, 2011). Oligodendrocytes are particularly susceptible to cell death following a brain or spinal cord injury (Almad et al., 2011), and the replacement of these cells could support the remaining damaged axons through re-myelination that would help restore electrical conduction in the axons.

Axons release neurotransmitters that influence oligodendrocytes at different stages in development through mediation of Ca^{2+}-dependent pathways (Butt, 2006). The gene *golli-mbp*, discovered in 1993 by Campagnoni et al., is important for regulating Ca^{2+} signaling in OPCs and oligodendrocytes through depolarization. The down-regulation of golli-MBP could inhibit oligodendrocyte depolarization resulting in Ca^{2+} dysregulation. The M3 enhancer in oligodendrocytes is responsible for maximal expression of *mbp* and *golli-mbp* (Dib et al., 2011). MBP is required for oligodendrocytes and Schwann cells to effectively myelinate their axons, but it is more highly expressed in the CNS (Dib et al., 2010). Dib et al. (2010) observed that deletion of the M3 enhancer results in the down-regulation of classic *mbp* as well as *golli- mbp* splice variants *in vivo*. This may inhibit the ability of OPCs to properly interact with their various growth factors.

While primary injuries are often sudden and unexpected, secondary injury mechanisms can be studied and eventually treated (Oyinbo, 2011). One mode of treatment under investigation is to transplant oligodendrocyte-fated progenitor cells into the spinal cord at various stages of injury to induce re-myelination and promote functional recovery. To further understand golli-MBP interactions *in vitro*, a model of wild-type (WT) and M3 enhancer knockout (M3KO) ESCs were treated with various growth factors and compared at various time points. Since growth factors, specifically FGF and PDGF, play similar roles in the development of oligodendrocytes in the CNS and in the differentiation of ESCs to OPCs, the knockdown of golli-MBP may, in turn, cause an inhibition of growth factor receptor pathway signaling, due to Ca^{2+} efflux dysregulation.

Methods

C57/BL6 Mouse Embryonic Fibroblast (mEF) Culture Conditions

The WT and M3KO C57/BL/6 mice were provided as gifts from the Peterson Lab at the Royal Victoria Hospital (McGill University). Three Cultures of both M3KO and C57/BL/6 mouse embryonic fibroblasts mEFs were grown in parallel. mEFs were provided by Nationwide Children's Hospital. DMEM supplemented with 10% fetal bovine serum (FBS) and 100 U penicillin / 100 µg/ ml streptomycin were used to feed the mEF cultures. MEFs were quickly thawed in a 37°C water bath directly from liquid N_2 and suspended in the mEF media under a biosafety cabinet hood (~$1x10^6$ cells per 10 cm plate). The cells were plated on 10 cm surface modified culture dishes and incubated at 37°C and 5% CO_2 until confluent.

C57/BL6 mEF Feeder Layer Formation

0.25% gelatin plates act as a foundation for the C57/BL6 mEF feeder layer. Gelatin plates were created by dissolving 25 mg gelatin in 10 ml dIH_2O in a 37°C water bath. Once it is completely dissolved,

the gelatin is sterilized using a 0.4 µm syringe filter. The gelatin was then coated onto cell culture plates with 10 ml per 10 cm plate overnight at 37°C.

The media from the mEF culture was aspirated and washed twice with Dulbecco's phosphate-buffered saline (DPBS). Immediately after aspirating the DPBS, 4 ml of 0.25% Trypsin was added per 10 cm plate. The plate was then incubated at 37°C until the mEFs lifted from the substratum. The mEFs were transferred into 10% FBS in DPBS with a disposable pipet and centrifuged for five minutes at 1000 rpm and 25°C. The supernatant was gently aspirated, replaced with fresh mEF media, and triturated. The gelatin was aspirated, and the plates were rinsed twice with DPBS. The cells were plated with mEF media onto the gelatin coated plates. Once the mEFs were confluent, they were inactivated with 10 µg/ml mitomycin C in mEF media and incubated at 37°C for three hours. After three hours, the mitomycin C was aspirated from the fibroblasts and washed three times with DPBS. The plates were then replaced with fresh mEF media and incubated overnight at 37°C and 5% CO_2.

Undifferentiated Embryonic Stem (ES) Cell Expansion

C57/BL6 WT ES cells were purchased from American Type Culture Collection (ATCC). Undifferentiated M3KO ES cells were produced by the ES care facility in Nationwide Children's Hospital's. DMEM/F12 supplemented with 10% ES-FBS, 100 U penicillin/ 100 µg/ ml streptomycin, 1% non-essential amino acids, 2 mM Glutamine, 55 µM β-Mercaptoethanol, and 10 ng/ml LIF were used to feed the ES cultures. ES cells were quickly thawed in a 37°C water bath, directly from liquid N_2. MEF media was aspirated from the feeder layer and washed twice with PBS. The ES cells were suspended in ES-LIF media (~$1x10^6$ cells / 10 cm plate) and transferred onto the feeder layer culture plates. The plates were incubated at 37°C and 5% CO_2. The ES-LIF media was changed daily until the formation of large ES colonies.

Feeder Layer mEF Removal

After the formation of large ES colonies, media was aspirated from the cultures and the plates were washed twice with DPBS. 4 ml 0.25% trypsin was added per 10 cm plate and incubated at 37°C until the ES colonies lifted from the substratum. Once the colonies lifted, they were transferred using a disposable pipet with the tip cut off into 10% FBS in DPBS. The cells were centrifuged for 5 minutes at 1000 rpm and 25°C. The ES colonies were gently re-suspended in the 10 ml ES-LIF media and poured onto 10 cm surface treated culture plates. The plates were incubated at 37°C and 5% CO_2. The ES-LIF media was changed daily until large ES colonies re-formed.

Embryoid Body (EB) Formation and Culturing

EBs are non-adherent, 3-dimentional ES cell spheres that are used for differentiation into neuronal lineages. To establish the formation of EBs, large ES colonies were left to re-form on the feeder layer. 4 ml 0.25% Trypsin was added per 10 cm plate and the cells incubated at 37°C until the ES colonies lifted from the substratum. Once the colonies lifted, they were transferred using a disposable pipet with the tip cut off into 10% FBS in DPBS. The tips of the pipets were cut off to provide more surface area for the colonies to pass through with little disruption. The cells were centrifuged for 5 minutes at 1000 rpm and 25°C. The ES colonies were then gently re-suspended in serum-free, ES-knockout serum replacement (KSR) media (DMEM/F12, 20% KSR (Invitrogen), 100 U penicillin/ 100 µg/ml streptomycin, 1% non-essential amino acids, 2 mM Glutamine, 55 µM β-Mercaptoethanol) and poured onto low-adhesion petri dishes. The petri dishes were incubated at 37°C and 5% CO_2. The media was changed daily.

To change the suspension culture, the EBs were transferred to a 15 ml tube using a plastic pipet with the tip cut off. The EBs were allowed to gravity settle. The media was gently aspirated and EBs were re-suspended in fresh media by gently inverting the tube. The re-suspended EBs were poured into a fresh petri dish. From day four to seven, 0.2 µM of retinoic acid was added to the petri dishes to induce neural crest formation as well as the formation of the neuroectoderm. From day five to seven, 1µM of

purmorphamine, a sonic hedgehog (SHH) inducer, was added to further induce neural progenitor formation. On day six, KSR was removed and the plates were supplemented with 1% N2 (Invitrogen), 0.2 µM Retinoic Acid, and 1µM purmorphamine.

Adherent OPC Culture

On day seven, surface modified culture dishes were pre-coated with 0.01% polyornithine and 5 µg/ml laminin in DPBS. The dishes were incubated overnight at 4°C. The 0.01% polyornithine and 5 µg/ml laminin in DPBS was then aspirated and washed twice with DPBS. The EBs were transferred to a 15 ml tube and allowed to gravity settle. The supernatant was gently aspirated. The EBs were re-suspended in 1% N2 media (minus RA and purmorphamine) and supplemented with 10 ng/ml basic fibroblast growth factor (bFGF). The media was changed daily as the EBs were allowed to gravity settle, and become adherent. After four days, the N2/FGF media was further supplemented with 10 ng/ml epidermal growth factor (EGF). Treatment with FGF/EGF for prolonged time induced the EBs to differentiate and develop oligodendrocyte precursor cell (OPC) morphology. Four days later, the FGF and EGF were removed and the N2 media was supplemented with 10 ng/ml PDGF for three days.

RNA Extraction

RNA was extracted at three different phases of ES differentiation into OPCs. RNA was extracted from the control group after a large embryonic body formation, then from the EBs treated with FGF, and the EBs treated with PDGF. Embryoid body suspension cultures, or adherent EB cultures, were lysed with 500 mL TRIzol® reagent (Invitrogen), and ground using sterile, disposable pestles. The TRIzol® suspensions were extracted with 200 mL of chloroform, and the resulting aqueous phases transferred to RNEasy (Qiagen) columns. The RNA was purified as per the manufacturer's protocol, and quantitated.

Reverse Transcription Reaction

10 mL of RNA (~10 mg) was combined with 2.5 U of DNAse (Roche), and incubated for 30 mins at 37°C to remove any contaminating gDNA. 5 U of Moloney's Murine Leukemia Virus (M-MLV) (Invitrogen) reverse transcriptase, 1 mM dithiothreitol (DTT), 0.1 mM anchored oligo-dT primer (Invitrogen), and 10mM nucleotides were combined with the reaction. The RT was allowed to incubate overnight at 37°C, and the resulting cDNA purified using QIAquick® (Qiagen) columns, as per manufacturer's protocol. The purified cDNA was quantitated by spectrophotometry.

Quantitative Real-Time PCR

50 mL of 15 ng/µL cDNA was combined with 100 µL RT2 CYBR green (Qiagen) and 50 µL H2O to produce a master mix for 10 reactions, in triplicate, for each cell culture condition. 20 µL (75 ng cDNA) of the master mix was added to the plate wells containing primers for the eight signature target genes (*Nanog, Stat3, Gli2, Pax6, Hspg4, Olig2, mbp, gfap*) and two endogenous controls (*gapdh* and *actb*) in a pre-made signature gene PCR 96-well plate (Qiagen). The PCR reactions were run through forty cycles, and the threshold cycles (CT) values for each reaction determined using the supplied qRT-PCR software (ABI).

Each gene product, relative quantity was compared using the ΔΔCT method. Briefly, the CT values for each gene product were, first, set relative to the endogenous control by:

$$\Delta CT = CT, gene - CT, control \quad (1)$$

The relative ΔCT values were then compared between the FGF-treated and the untreated EB samples for each ES cell origin (wild type and M3KO):

$$\Delta\Delta CT, gene = 2^{-(\Delta CT, FGF - \Delta CT, EB)} \quad (2)$$

All triplicate gene values were compared using the above equation (2), and the average ΔΔCT value computed. To determine significance between the wild-type and M3KO groups, student t-Tests were performed for each gene's replicate ΔΔCT values.

Results

1. **Cell Culture**

a) Embryonic stem cell cultures

There were no large morphological differences observed between the WT and M3KO cultures at the adherent embryonic stem cell culture stage. Both WT and M3KO ESCs grew well *in vitro*. The WT ESCs grew into tight, compact balls whereas the M3KO ESCs were not as compact or confluent. Both cultures quickly adhered to the mEF monolayer and proliferated until they were approximately 80% confluent.

b) Non-adherent embryoid body cultures

After the formation of large ES colonies, the mEF feeder layer was removed and the large ES colonies were left to re-form. Both the WT and M3KO cultures grew into EBs in the same timeframe. Both cultures were then trypsinzed and transferred into KSR media where they successfully expanded into large, non-adherent EBs (Figure 1). The WT and M3KO cultures were then treated with retinoic acid and purmorphamine to sequentially induce their transition into neuronal progenitor cells. Upon the introduction to these supplements, we began to observe that some WT cells were prematurely adhering to the petri dish. The M3KO cultures did not experience this adhesion phenomenon.

c) Adherent embryoid body cultures supplemented with FGF

The WT and M3KO cultures were transferred to 0.01% polyornithine/laminin plates, and re-suspended in N2 media supplemented with 10 ng/ml bFGF to induce OPC differentiation.

The EBs were allowed to gravity settle and become adherent. The WT EBs immediately began to adhere, however the M3KO EBs failed to settle. By the eighth day, when both bFGF and EGF were to be replaced with PDGF, we observed a large amount of differentiation in WT EBs. We noticed the cells began migrating away from the EB as they differentiated, leaving room for more cells to differentiate from the edges of the EB (Figure 1). However, the majority of M3KO EBs were still non-adherent and undifferentiated.

d) Embryoid body cultures treated with PDGF

The growth factors FGF and EGF were removed and replaced with 10 µg/ml PDGF. The WT cultures were completely adherent and differentiated robustly. The WT EBs generated OPCs that consisted of cells with two or three processes extending from opposing sides of the cell body (Figure 1). Even with the polyornithine-laminin treated plates and treatment with PDGF, the majority of M3KO EBs still failed to adhere. The EBs that did adhere failed to differentiate and migrate from the EBs.

2. Q-RT PCR

The ΔΔCT compared FGF treated WT EBs with untreated WT EBs and FGF treated M3KO EBs with untreated M3KO EBs. The ES marker, Nanog, was significantly up-regulated in the FGF treated WT EBs compared to the untreated EBs with a ΔΔCT value of 2.1 (Figure 2). The glial marker Olig2, normally expressed in mature oligodendrocytes, was significantly up-regulated in the FGF treated WT EBs compared to the untreated EBs. Glial markers NG2 and MBP had ΔΔCT values of 0.3 and 0.5 respectively, thus were significantly down-regulated in the FGF treated M3KO EBs compared to the untreated M3KO EBs. A student t-test examination indicated a significant level of variance between the WT and M3KO EBs for glial markers Pax6 (p-value= 0.04) and NG2 (p-value= 0.01). The ES marker Gli2 was non-detectable for both WT and M3KO EBs. The glia marker MBP in the WT EBs was also non-detectable.

Figure 1

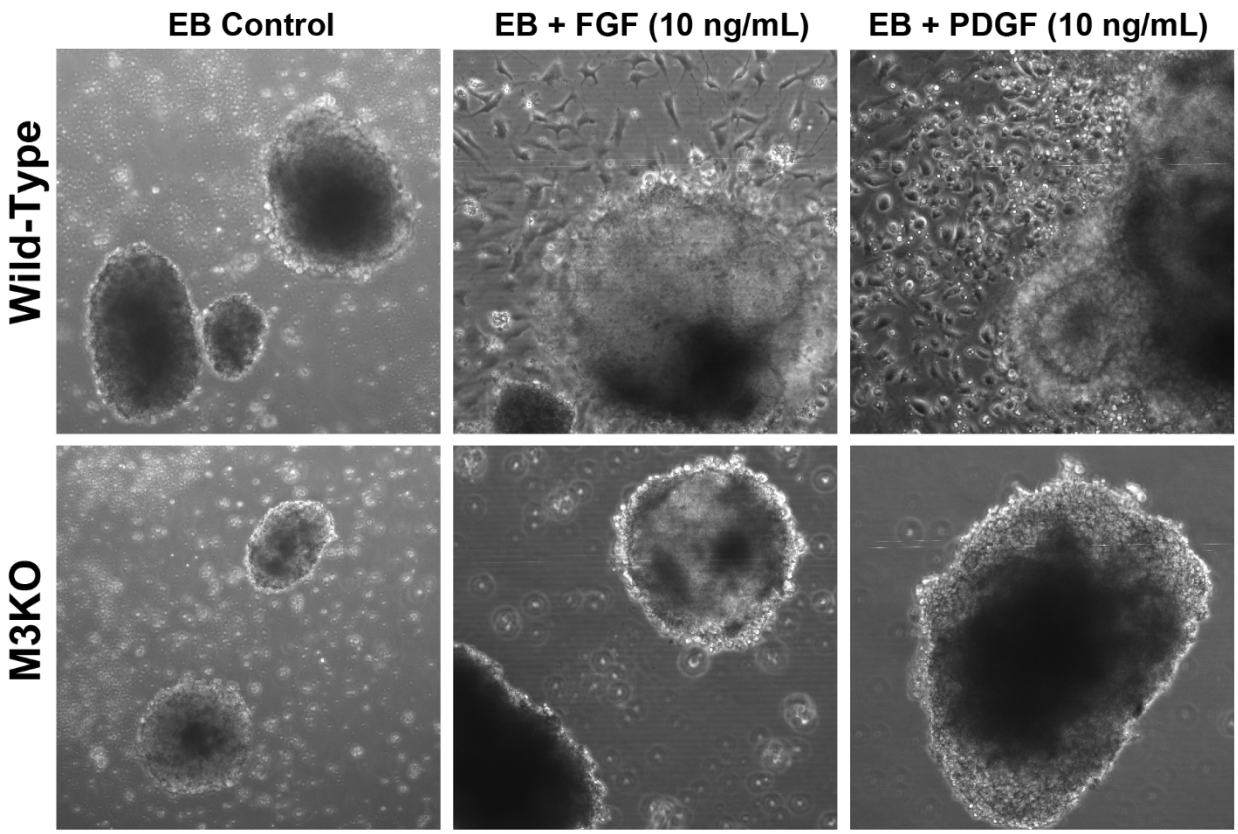

Figure 1. M3KO EBs do not differentiate in response to the growth factors FGF and PDGF. WT and M3KO EBs were plated onto poly-L-ornithine and laminin treated plates. In the untreated controls, the WT EBs grew larger and more robust than M3KO EBs. Upon treatment with 10 ng/µl FGF for three days, WT EBs became adherent, and the differentiating cells migrated from the EB. Treatment with 10 ng/µl PDGF for three days increased WT differentiation and promoted an OPC phenotype. The growth factor supplementation with FGF and PDGF seemed to have no effect on the M3KO EBs, in that there was no observed adhesion nor differentiation.

Figure 2

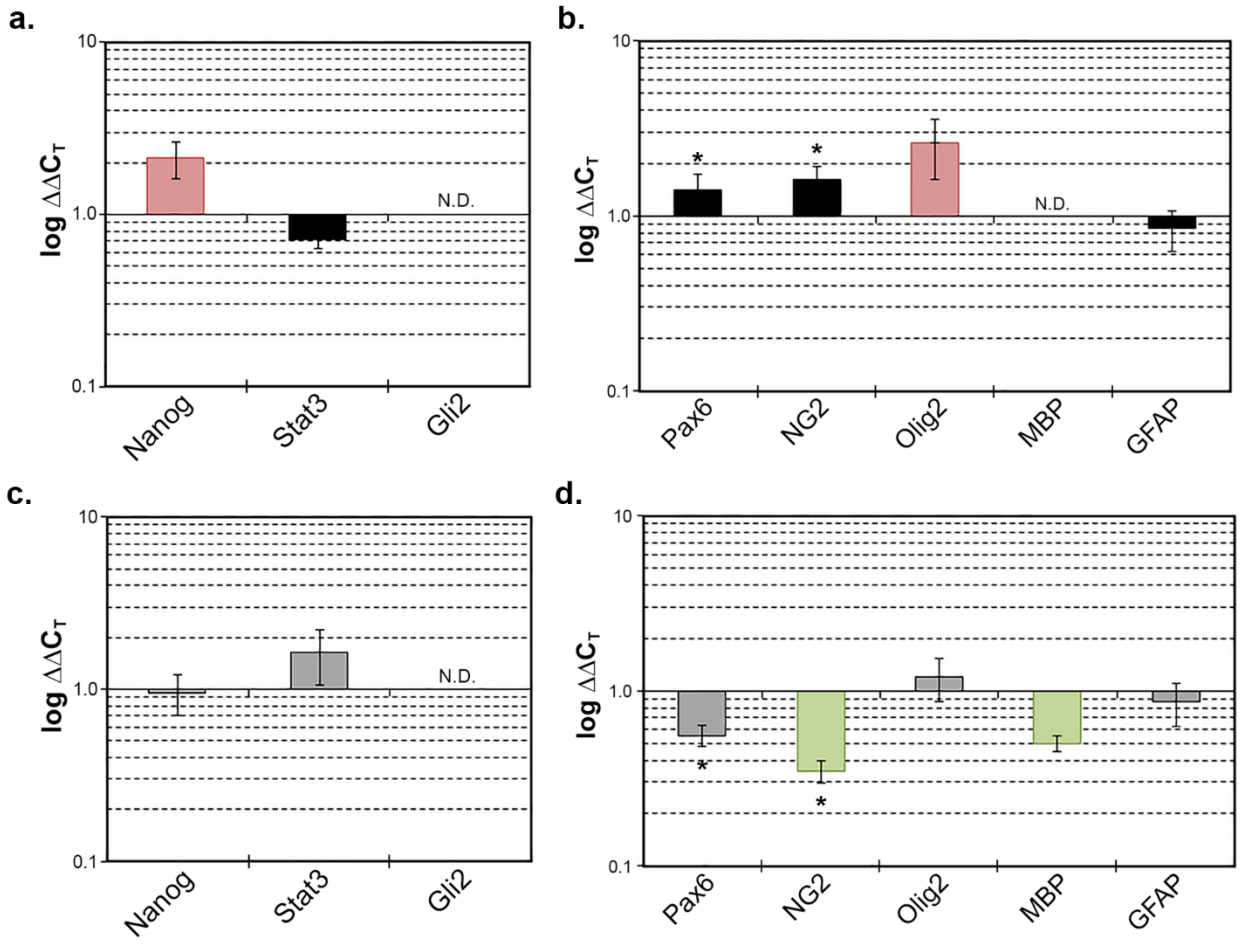
a.
b.
c.
d.
log ΔΔC_T
10
1.0
0.1
N.D.
Nanog
Stat3
Gli2
Pax6
NG2
Olig2
MBP
GFAP
*

Figure 2. Glia marker gene products were significantly down-regulated in M3KO EBs. 75ng of RT product from the WT and M3KO extractions were analyzed by qRT. The ΔΔCT levels of significance were $0.5 \geq \Delta\Delta CT \geq 2.0$. Red indicates the significant up-regulation of gene markers for ΔΔCT, green represents the significant down-regulation of gene markers for ΔΔCT, black indicates the non-significant ΔΔCT values of WT EBs and grey indicates the non-significant ΔΔCT values of M3KO EBs. The asterisks represent a level of significance at $p \leq 0.05$ for the student t-test examination. The WT ES gene marker, Nanog, was significantly upregulated at ΔΔCT= 2.1 in response to prolonged FGF treatment relative to no treatment in contrast to the ES gene markers STAT3 and Gli2 (A). The WT Glial gene marker, Olig2, was significantly upregulated at ΔΔCT= 2.6 (B). The ΔΔCT values of the M3KO ES gene markers, in response to prolonged FGF treatment relative to no treatment, were not significant (C). The M3KO Glial gene markers for NG2 and MBP were significantly downregulated at ΔΔCT= 0.3 and 0.5 respectively (D).

Discussion

Previous studies have shown that implantation of OPCs into injured spinal cords could generate mature oligodendrocytes to restore axon myelination and efficient electrical conductance between synapses (Thuret et al., 2005). Initially, we planned to implant fated pre-OPC WT and M3KO ESCs into mice with spinal cord injuries. The fated pre-OPCs are plastic, late-stage ESCs that would likely survive and adapt to their change in environment by responding to growth factors released from the injury site. We hypothesized that the pre-OPCs would become OPCs and then mature into myelinating oligodendrocytes. Further, since many differentiation processes are Ca^{2+} dependent and golli-MBP is known to modulate many intracellular events in OPCs through store-operated and voltage-gated calcium channels (VOCC) (Chew et at., 2010), we intended to use the M3KO model to further investigate the role of golli-MBP in Ca^{2+} dysregulation and its effects on remyelination post-injury.

In adherent EB cultures, bFGF was added to promote OPC morphology and the addition of PDGF further induced the EBs to differentiate and commit to the oligodendrocyte cell lineage. While the WT EBs adhered and differentiated robustly during cell culture, we failed to replicate these results in the M3KO EB cultures even with the addition of the various growth factors (Figure 1). These results suggest that the knockdown of golli-MBP may have resulted in the dysregulation of voltage-gated and SOCCs of the EBs that lead to the inhibition of growth factor signaling pathways. It is still unknown where this Ca^{2+} dysregulation stems from. One possibility is that the efflux of Ca^{2+} activates calmodulin kinase (CamK), which in turn is known to phosphorylate Erk1/2 (Hudmon and Schulman, 2002). Additionally, Ca^{2+} can also actuate protein kinase C (PKC), which acts upstream of Erk1/2 by stimulating Ras and thus initiating the MAP kinase signaling cascade. The additive effects of these two kinases converging at Erk1/2 may cause the over-activation of down-stream effectors, particularly transcription factors.

The Erk1/2 MAP kinase signaling cascade is critical for the termination of the ES cell self-renewal program to induce differentiation (Ying et al., 2008). In a process termed feedback inhibition, Erk1/2 can

inhibit its own phosphorylation by binding to its upstream effectors, MEK1/2, Grb, and SOS. Erk1/2 can bind to MEK1/2, producing a barrier that physically inhibits the binding and phosphorylation of the entire Raf family (Dhillon et al., 2007). Binding to Grb and SOS would inhibit them from binding to the receptor tyrosine kinase dimer through a negative feedback loop.

In another aspect of feedback inhibition, Erk1/2 can also enter the nucleus to phosphorylate various transcription factors, such as Elk-1 and AP-1. The transcription factors are then able to bind the promotors of dual specificity phosphatase 1 (DUSP1) and dual specificity phosphatase 6 (DUSP6) which act as negative feedback regulators. The DUSPs can directly dephosphorylate Erk1/2 in the cytosol, or inhibit phosphorylation of transcription factors by Erk1/2 in the nucleus. In the M3KO EBs, the continuous dephosphorization of Erk1/2 may cause the down-regulation of genes that are necessary for ES differentiation. Additionally, the over-expression of the DUSPs could cause not only the de-phosphorylation of Erk1/2, but other MAP Kinase pathways as well, such as the p38. The p38 MAP Kinase pathway is also known to regulate OPC development into oligodendrocytes (Chew et al., 2010). The de-phosphorylation of this pathway by DUSP6 may possibly inhibit ES differentiation and may inhibit the M3KO EBs from progressing into the OPC lineage.

Conversely, the over-activation of ERK1/2 by calcium-dependent kinases may also cause Erk1/2 to phosphorylate transcription factors it does not normally act upon, under different cellular contexts. This, in turn, may cause the transcription of genes outside of its normal cell-type, including genes that inhibit differentiation. The activation of these various genes may explain the significant downregulation of the M3KO ES signatures (Figure 2d).

In normal ESCs, Nanog is part of the feedback loop with Oct4 and Sox2 to regulate proliferation and self-renewal. The ES marker, Nanog, was significantly upregulated in the WT EBs treated with FGF compared to the untreated WT EBs (Figure 2a). Although this result may be an anomaly, it suggests that FGF promotes proliferation in the WT EBs as well as differentiation. We expected Nanog to be down-

regulated in the FGF supplemented WT EBs due to the differentiation we observed from the WT EBs *in vitro*. Nanog was possibly up-regulated in the WT EBs treated with FGF because both the EBs and their differentiating cells were included together in the ΔΔCT calculations. This indicates that FGF may be serving two functions at once. Since the majority of the cells were undifferentiated and still packed together inside of the EBs, the significant up-regulation of Nanog may indicate that FGF regulates the proliferation of the undifferentiated cells within the WT EBs as well as the differentiation stemming from the EB surface. There seems to be no difference in the expression of Nanog between FGF treated and untreated M3KO EBs (Figure 2c). This may show that, unlike the WT, the addition of FGF neither promotes proliferation nor differentiation in the M3KO EBs.

Pax6, NG2, and Olig2 were all upregulated in the WT with FGF treatment (Figure 2b). However, the significant upregulation of Olig2, a glial marker for oligodendrocytes, in the FGF supplemented WT EBs suggest that the EBs were differentiating and beginning to express OPC morphology. Due to Pax6 being a general CNS marker, its significant upregulation in the WTs compared to the M3KOs indicates that the WT EBs were beginning to develop a neuronal phenotype but were still plastic enough that cell fate was not yet decided. This may be due to the additions of retinoic acid and purmorphamine promoting neural crest and neural progenitor formation. The differentiating cells still had the choice to mature into neurons or oligodendrocytes. The significant upregulation in WT EBs compared to M3KO EBs for NG2 suggests that the FGF supplementation in the WT induced cell commitment to differentiate into OPCs. The non-detection of MBP in the WT EBs suggests that the EBs were OPCs and do not have an oligodendrocyte morphology.

We hypothesized that FGF supplementation would have no effect on the M3KO EBs due to an inhibition of signaling in the growth factor receptor pathway. However, we found that Pax6 was downregulated in the FGF supplemented M3KO EBs (Figure 2d). When compared to WT expression levels, both NG2 and MBP glial markers were significantly downregulated in the M3KO EBs with FGF

treatment as well. This suggests that the M3KO EBs with FGF supplementation are not differentiating since the down-regulation of Pax6, NG2, and Olig2 indicate that they do not express an OPC morphology or phenotype. These results may signify that knock-down of golli-MBP, and the subsequent irregular Ca^{2+} signaling, may have an effect on the developmental dysregulation from M3KO ESCs into EBs, through the mechanisms mentioned previously.

Initially, we planned to use ESCs in order generate a pure culture of OPCs to implant into mice with traumatic spinal cord injuries. We hoped to use our M3KO mouse model, in this study, to investigate the effects of the knock-down of golli-MBP on Ca^{2+} *in vivo*. We were not able to accomplish this since the unforeseen side-effects of the golli-MBP knock-down resulted in the inability of the M3KO EBs to differentiate into OPCs (Figure 1), likely due to the dysregulation of SOCCs and VOCCs in the M3KO ESCs. The possibilities are that the elevation of Ca^{2+} levels in the cytosol may lead to the continuous phosphorylation of the MAP Kinase cascade, specifically Erk1/2. This in turn may lead to the up-regulation of unexpected differentiation inhibitory genes as well as the genes that are necessary for the typical differentiation program. The constant Erk1/2 activation may also cause the over-expression of DUSP1 and DUSP6 to not only de-phosphorylate Erk1/2, but de-phosphorylate other MAP Kinases, such as p38, as well. Previous studies have shown that golli-MBP is a vital component of VOCCs and SOCCs, and Ca^{2+} is central for many cell processes. Our data indicates that Ca^{2+} dysregulation due to the knock-down of golli-MBP negatively effects the proliferation, migration, and differentiation of ES cells into oligodendrocyte progenitor cells.

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